Two types of Hapaxomers

- Internal Palindromic Type II enzymes
  - e.g., *Sfi* I

```
G G C C N N N N^N G G C C
C C G G N^N N N N C C G G
```

- Outside Cutters (Type IIS)
  - e.g., *Sap* I

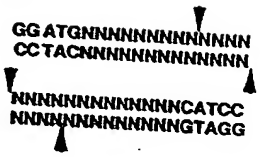
```
G C T C T T C N^N N N
C G A G A A G N N N N^
```

Fig 1

A


CAGNNNCTG
CAGNNNCTG

B


GGATGNNNNNNNNNNNNNN
CCTACNNNNNNNNNNNNNN
NNNNNNNNNNNNNNCATCC
NNNNNNNNNNNNNNGTAGG

Figs 2A-B

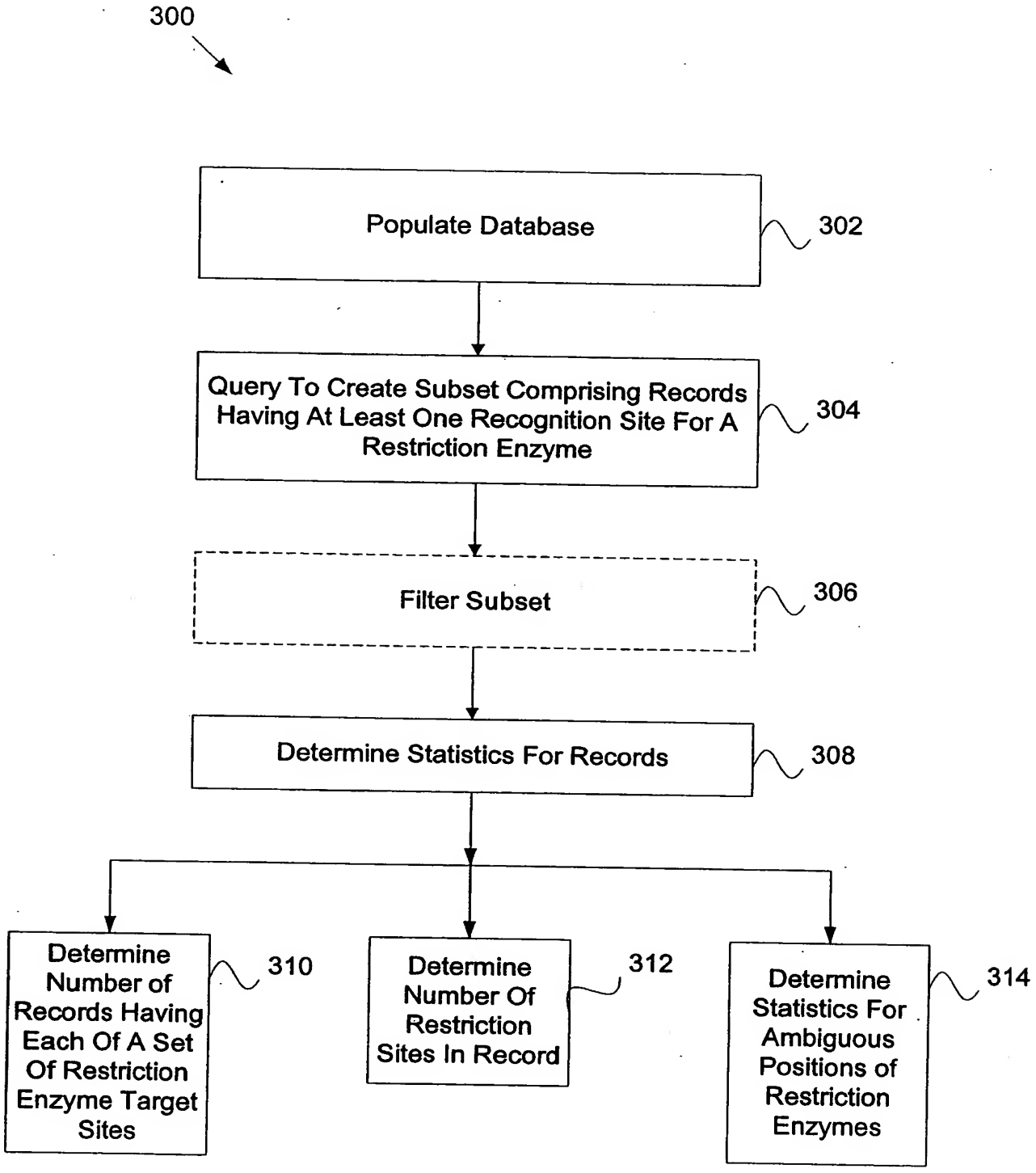

300
Populate Database
302
Query To Create Subset Comprising Records Having At Least One Recognition Site For A Restriction Enzyme
304
Filter Subset
306
Determine Statistics For Records
308
Determine Number of Records Having Each Of A Set Of Restriction Enzyme Target Sites
310
Determine Number Of Restriction Sites In Record
312
Determine Statistics For Ambiguous Positions of Restriction Enzymes
314

FIG. 3

	Sap I (0)	Sfi I (0)	Sfi I (0+1)	Sfi (0-2)	Sgf I/Pme I	Sap I (0)	Sap I (0+1)	Sap I (0-2)
Hs_Fna	56.72	85.93	96.72	98.91	97.45	56.72	84.67	94.82
Mgc	61.98	87.02	97.94	99.70	98.14	61.98	90.31	97.60
E coli	86.71	99.35	100.00	100.00	93.52	86.71	98.37	99.53
C elegans	65.93	99.46	99.97	100.00	98.55	65.93	90.38	97.19
S cerevisiae	80.02	99.15	99.98	100.00	97.17	80.02	96.68	99.29
Arabidopsis	70.83	99.56	100.00	100.00	97.57	70.83	93.37	98.63

Fig 4

7+ Cutters

Enzymes	Recognition Sequence	HsFna	MGC	Ec	Ce	Sc	At
AarI	CACCTGCNNNN^NNNN	7142	5355				1138
AbeI	*CC^TCA_GC not available*	*7970*	*5836*	*141*	*90*	*374*	*1833*
AscI	GG^CGCG_CC	515	336	152	10	13	26
AsiSI	GCG_AT^CGC	108	62	207	39	29	178
BbvCI	*CC^TCA_GC*	*7970*	*5836*	*141*	*90*	*374*	*1833*
CciNI	GC^GGCC_GC	1444	823	19	33	31	97
CpoI	*CG^GWC_CG*	*1119*	*781*	*347*			
CspI	*CG^GWC_CG*	*1119*	*781*	*347*			
CspBI	GC^GGCC_GC not available	1444	823	19	33	31	97
FseI	GG_CCGG^CC	1139	740	5	9	10	70
MabI	*A^CCWGG_T*						
MchAI	GC^GGCC_GC not available	1444	823	19	33	31	97
Mlu1106I	*RGGWCCY* not available						
NotI	GC^GGCC_GC	1444	823	19	33	31	97
PacI	TTA_AT^TAA	708	395	66	8	213	138
Pfl27I	*RG^GWC_CY* not available						
PpuMI	*RG^GWC_CY*						
PpuXI	*RG^GWC_CY*						
Psp5II	*RG^GWC_CY*						
PspPPI	*RG^GWC_CY*						
RsrII	*CG^GWC_CG*	*1119*	*781*	*347*			
Rsr2I	*CG^GWC_CG*	*1119*	*781*	*347*			
SanDI	*GG^GWC_CC*						
SapI	GCTCTTCN^NNN_	7260	4785	584	1296	1362	8870
SbfI	CC_TGCA^GG	2591	1802	60	13	66	251
SdaI	CC_TGCA^GG	2591	1802	60	13	66	251
SdiI	GGCCN_NNN^NGGCC not available	2214	1634	28	18	54	121
SexAI	*A^CCWGG_T*						
SfiI	GGCCN_NNN^NGGCC	2214	1634	28	18	54	121
SgfI	GCG_AT^CGC	108	62	207	39	29	178
SgrAI	CR^CCGG_YG						
Sse232I	CG^CCGG_CG not available	708	448	29	43	23	446
Sse1825I	*GG^GWC_CC* not available						
Sse8387I	CC_TGCA^GG	2591	1802	60	13	66	251
Sse8647I	*AG^GWC_CT* not available						
VpaK32I	GCTCTTCN^NNN_ not available	7260	4785	584	1296	1362	8870

Six Cutters

Enzymes	Recognition Sequence	HsFna	MGC	Ec	Ce	Sc	At
NruI	TCG^CGA	830	607	1070	558	507	2422
SplI	C^GTAC_G	701	449	498	263	549	1705
SnaBI	TAC^GTA	1080	621	435	165	885	2164
PvuI	CG_AT^CG	842	512	1000	705	537	3078
MluI	A^CGCG_T	1049	1019	976	295	337	1824
BglI	GCCN_NNN^NGGC	8827	6868	1333	469	750	2576
Ear I	CTCTTCN^NNN_						
BsrGI	TGTACA	6683	4551	442	760	1583	5450
XmnI	GAANN^NNTTC	8401	5850	1167	1652	2911	12141
SalI	G^TCGA_C	1515	944	463	792	856	3616
BamHI	G^GATC_C	6426	4305	438	1047	1238	6782
KpnI	G_GTAC^C	4098	2755	442	305	1317	2992
EcoRI	G^AATT_C	6536	4132	470	1346	2466	8244
XhoI	C^TCGA_G	3651	2402	156	800	737	7092
EcoRV	GAT^ATC	3789	2435	1378	919	2289	8419
	AAA^TTT	7484	5167	1008	1049	3843	8078
DraI	TTT^AAA	8455	6243	967	494	3018	6778

Fig 5

7+ Blunt Cutters

Enzymes	Recognition Sequence	HsFna	MGC	Ec	Ce	Sc	At
BstRZ246I	ATTT^AAAT	1204	648	55	38	379	317
BstSWI	ATTT^AAAT	1204	648	55	38	379	317
MspSWI	ATTT^AAAT	1204	648	55	38	379	317
MssI	GTTT^AAAC	297	173	71	9	152	490
PmeI	GTTT^AAAC	297	173	71	9	152	490
SmiI	ATTT^AAAT	1204	648	55	38	379	317
SwaI	ATTT^AAAT	1204	648	55	38	379	317
SrfI	GCCC^GGGC	1433	887	40	11	11	30

Hs_Fna 15740 entries
Mgc 12585 entries
Ec 4290 entries
Ce 3305 entries
Sc 6360 entries
At 27289 entries

Interrupted Palindromes

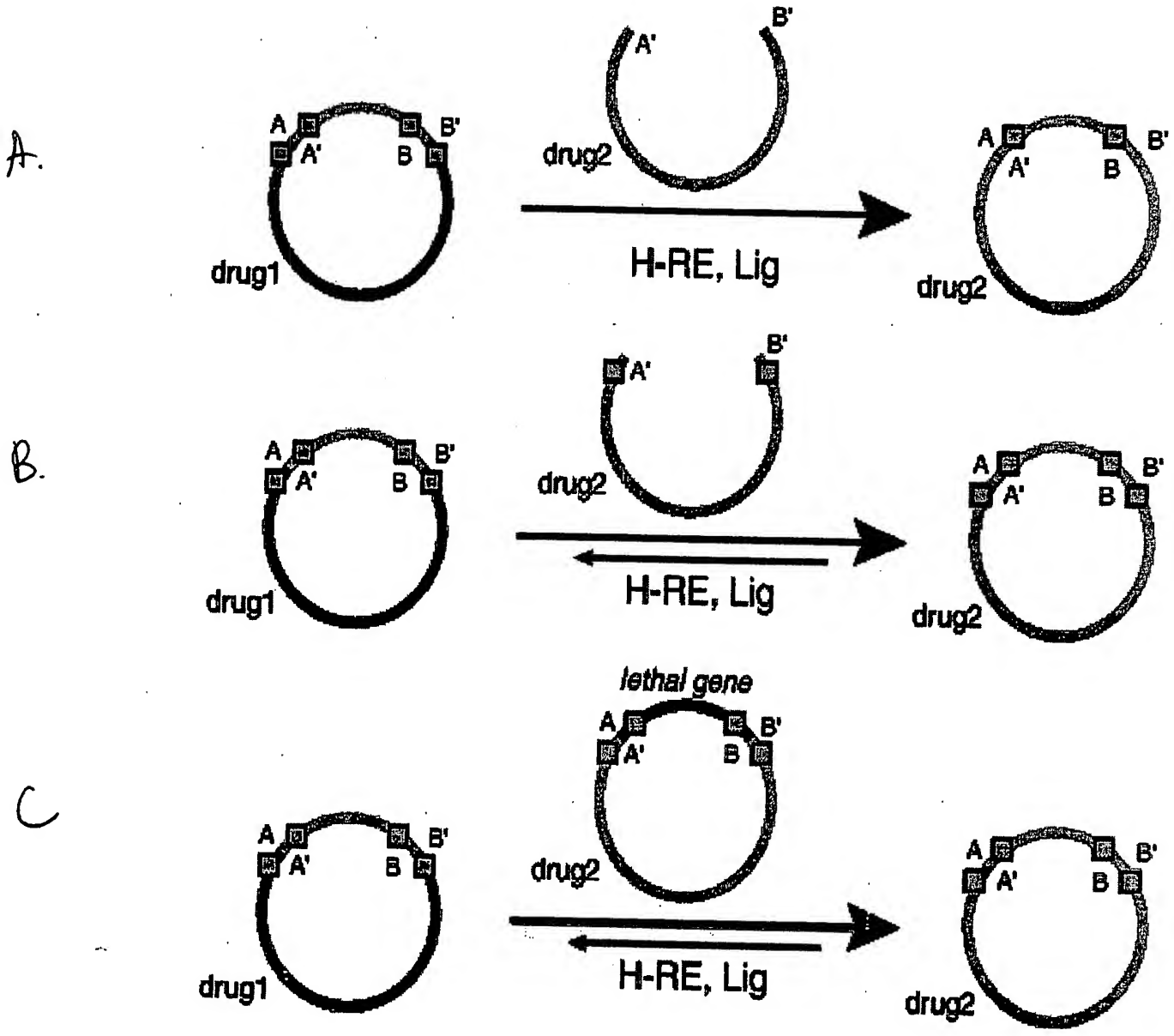


Fig 6

Sfi I

- How to make *Sfi* I "one way"
  - Methylases
  - *Bgl* I, not *Sfi* I sites, in Acceptor Vectors

```
G G C C N N N N^N G G C C
C C G G N^N N N N C C G G

  G C C N N N N^N G G C
  C G G N^N N N N C C G
```

  - Lethal genes in stuffer fragments

Fig 7

PCR Interrupted Palindrome Cloning Pathway

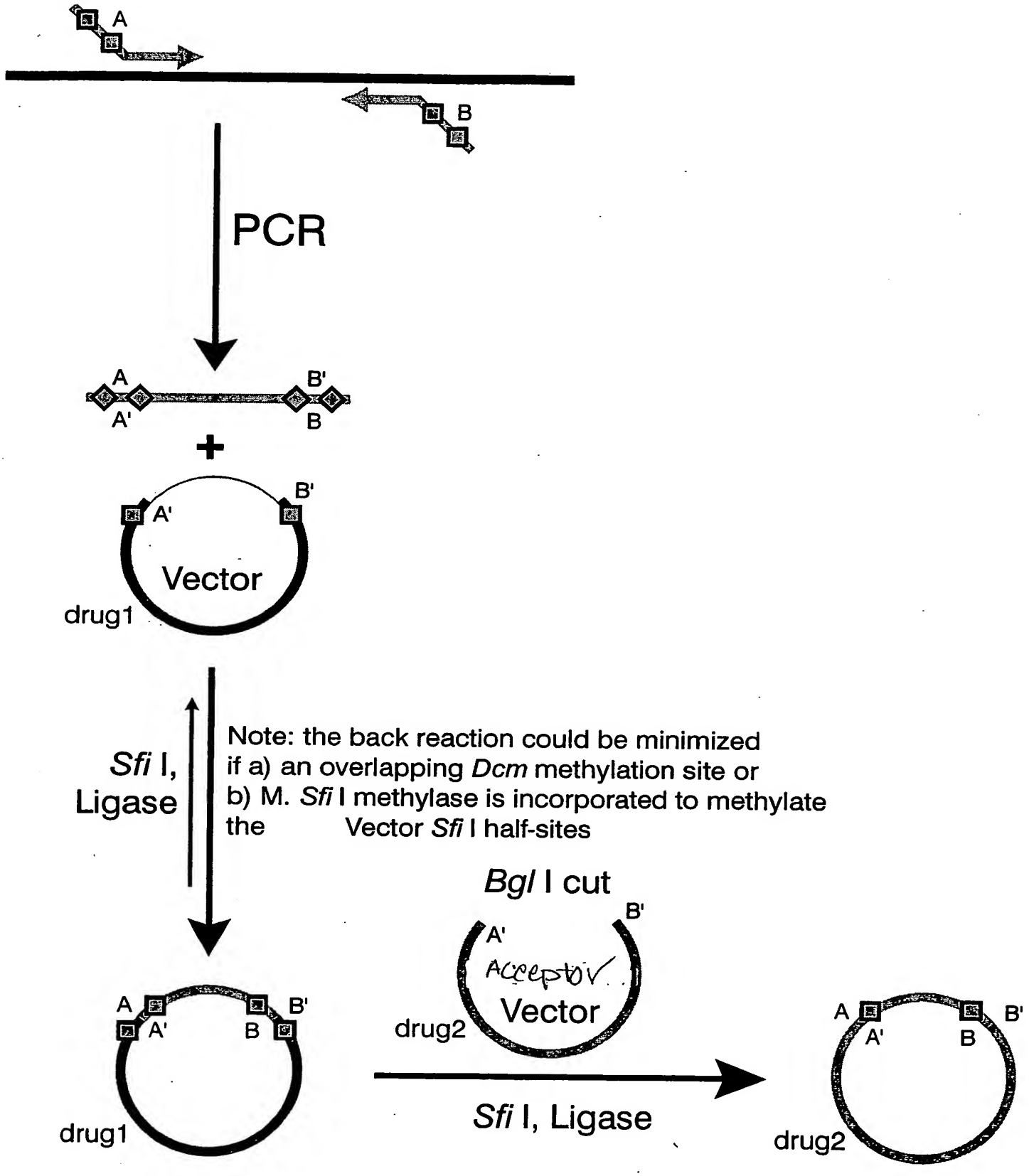


Fig 8

PCR Interrupted Palindrome Cloning Pathway 1A

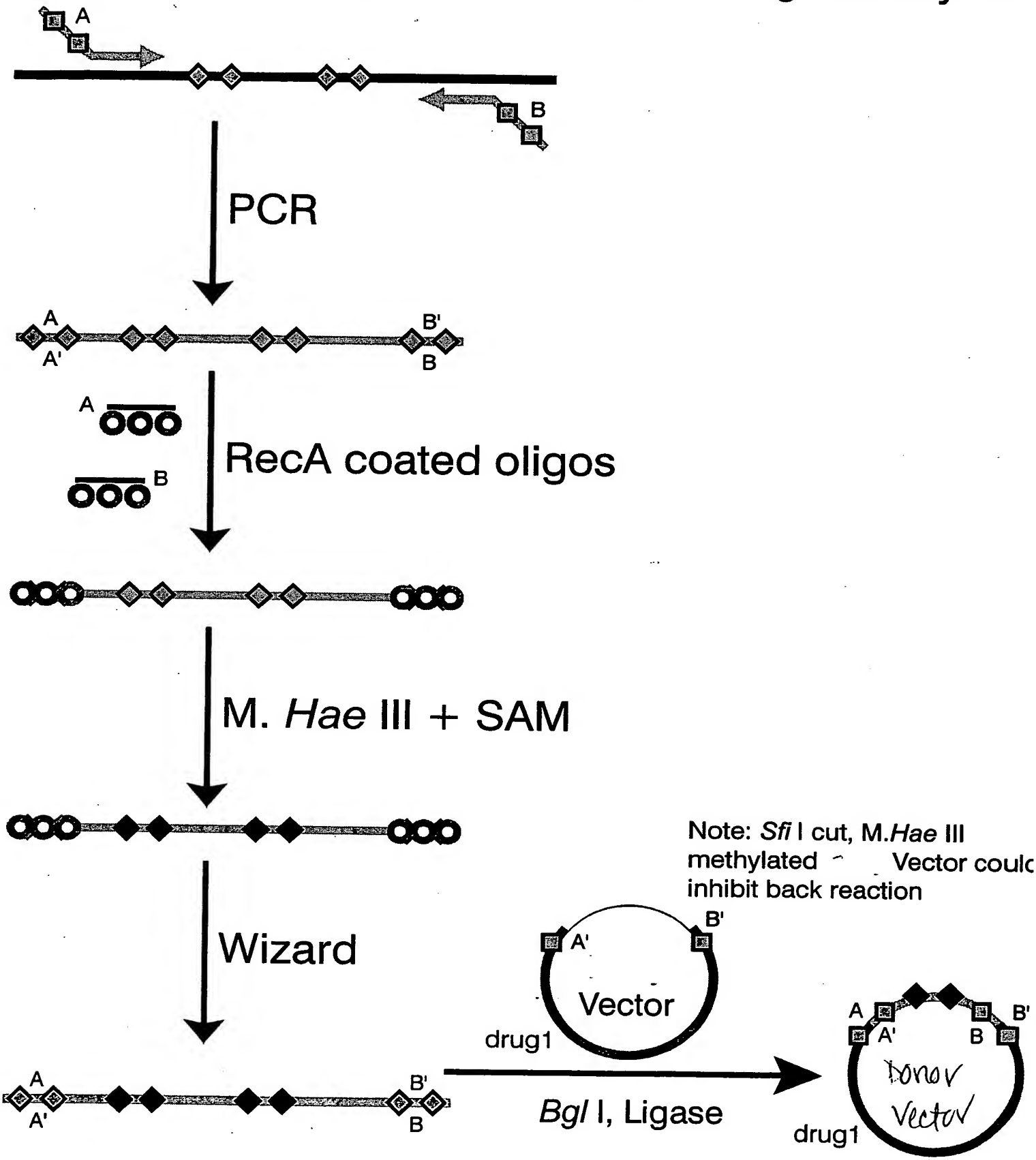


Fig 9A

PCR Interrupted Palindrome Cloning Pathway 2A

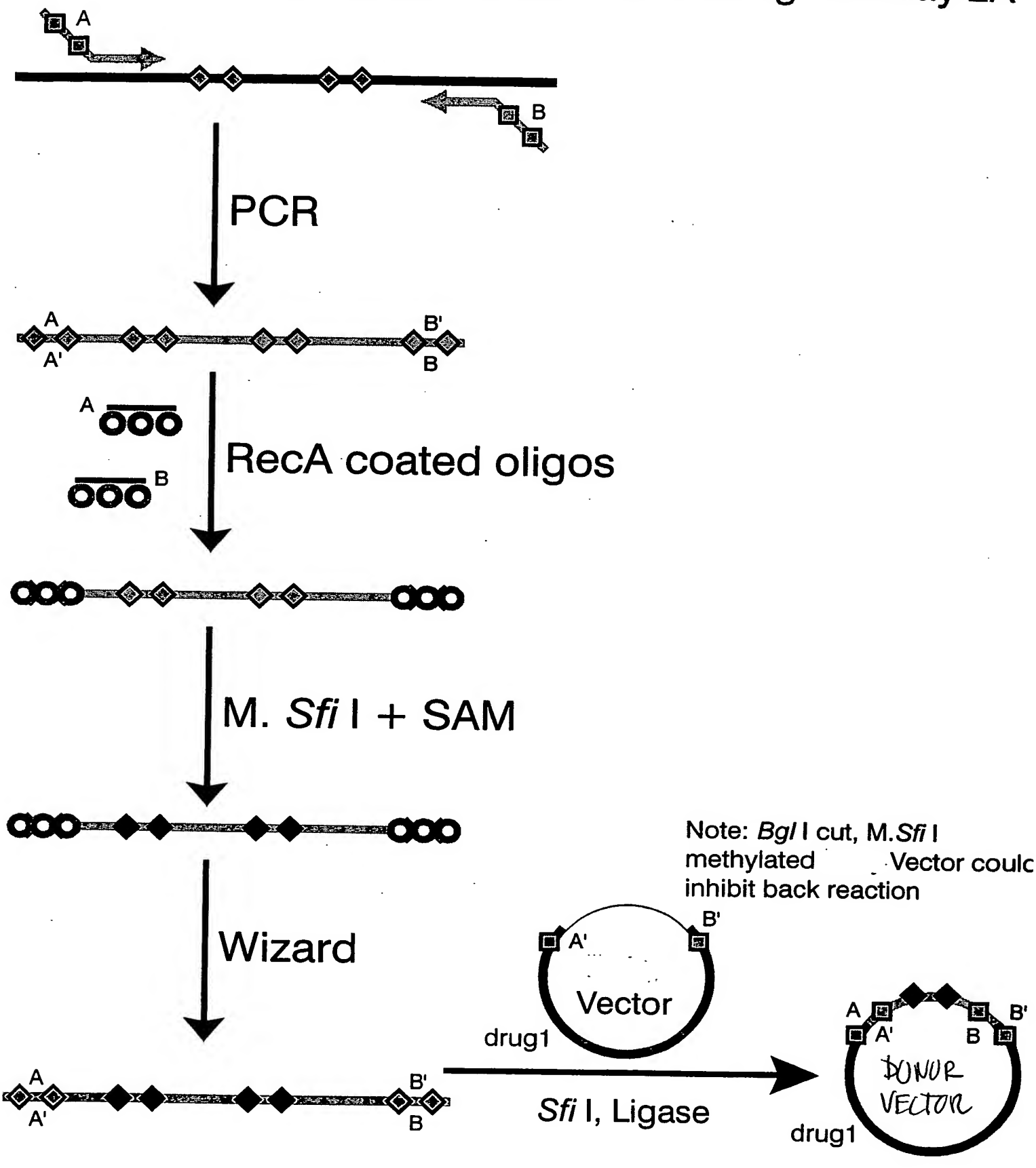


Fig 9B

PCR Interrupted Palindrome Cloning Pathway 1B

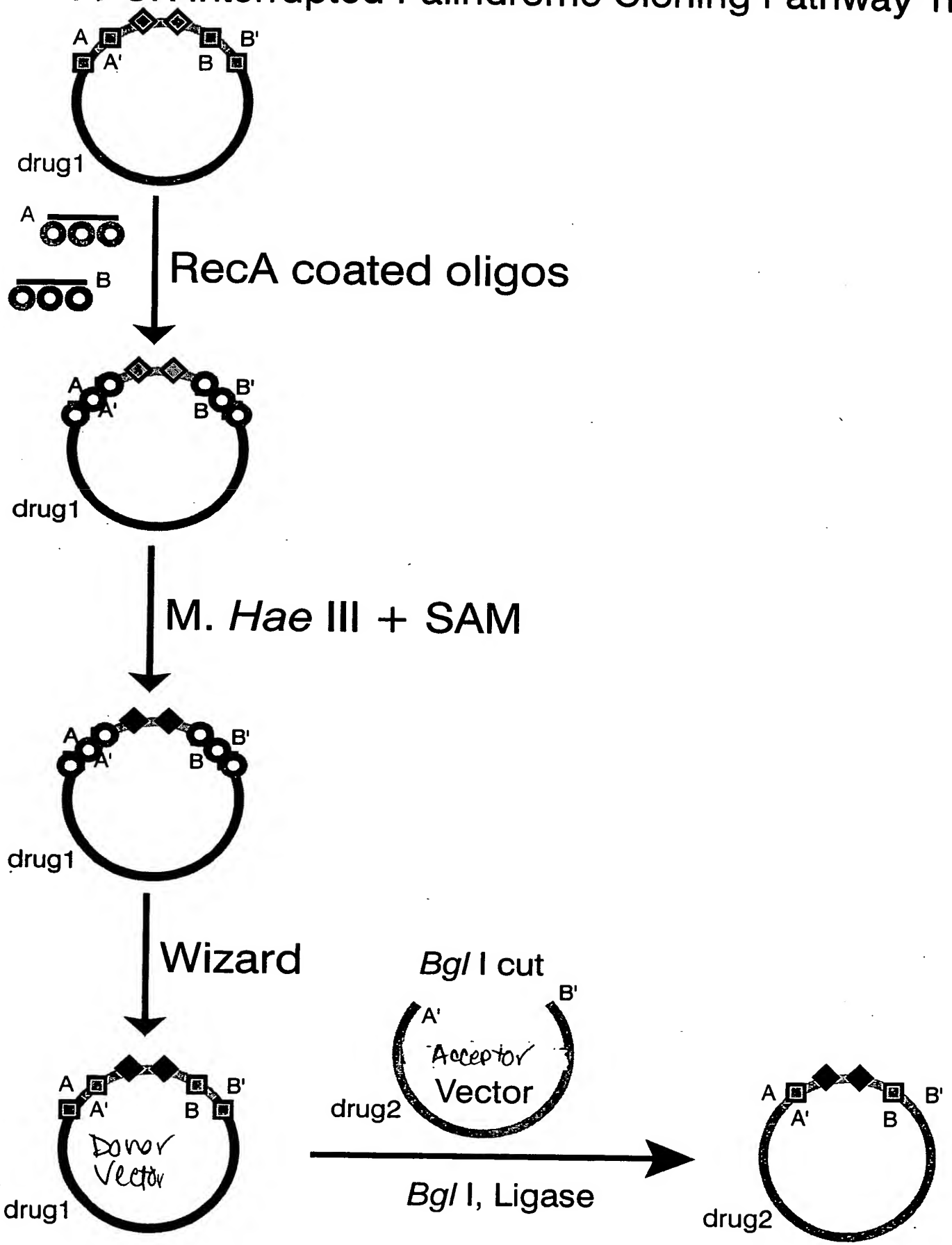


Fig 10A

PCR Interrupted Palindrome Cloning Pathway 2B

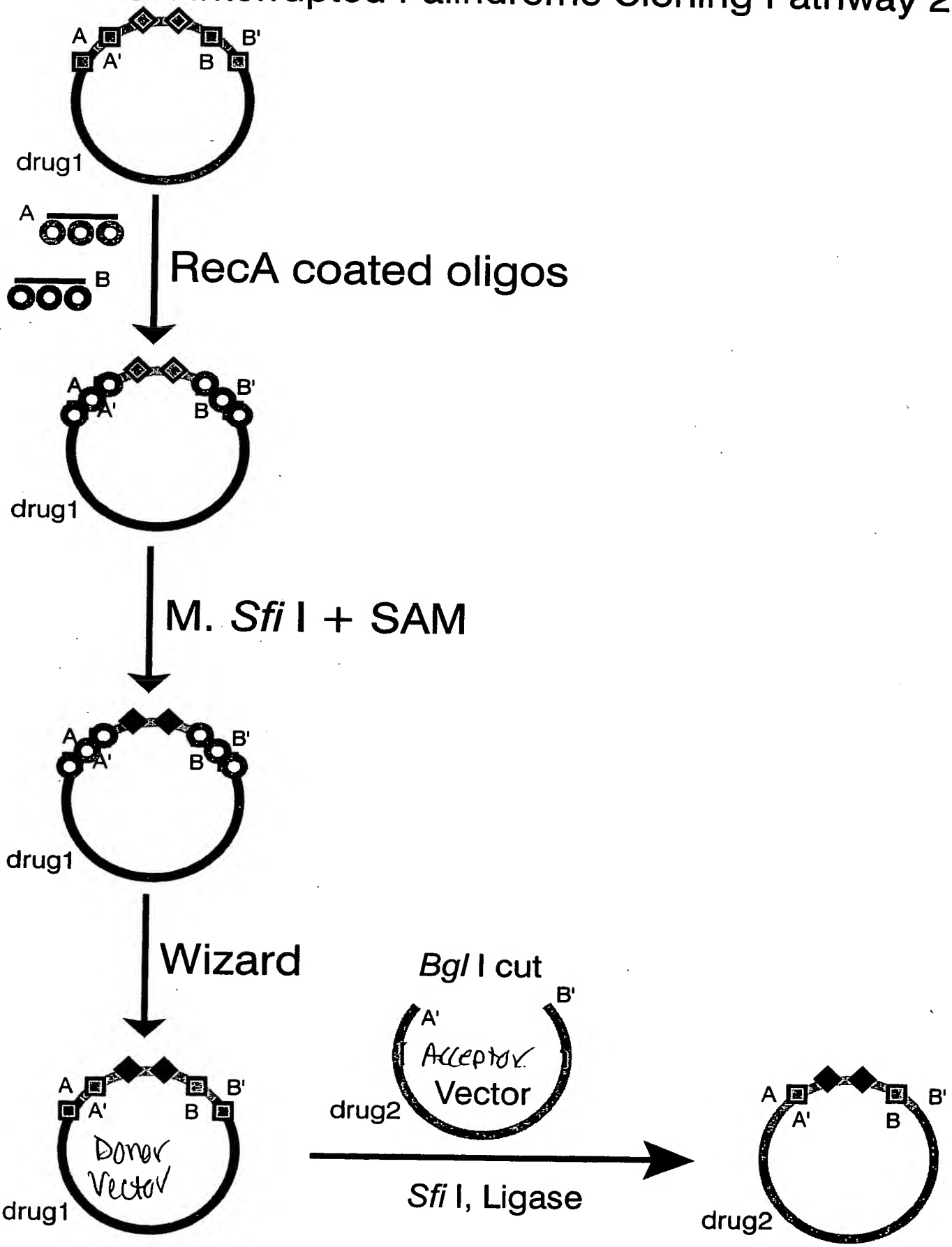


Fig 10B

REs that can make *Sfi* I one-way

3' 3b Overhang	Restriction Enzyme	Recognition Sequence
CNG	FmuI	G_GNC^C
CNG	PssI	RG_GNC^CY
CWG	Psp03I	G_GWC^C
GNC	BthCI	G_CNG^C
GSC	TauI	G_CSG^C
NNN	AlwNI	CAG_NNN^CTG
NNN	BglI	GCCN_NNN^NGGC
NNN	BsiYI	CCNN_NNN^NNGG
NNN	BstAPI	GCAN_NNN^NTGC
NNN	DraIII	CAC_NNN^GTG
NNN	MwoI	GCNN_NNN^NNGC
NNN	PflMI	CCAN_NNN^NTGG
NNN	RleAI	CCCACANNNNNNNNN_NNN^
NNN	SfiI	GGCCN_NNN^NGGCC

Fig 11

Outside Cutters (Type IIS)

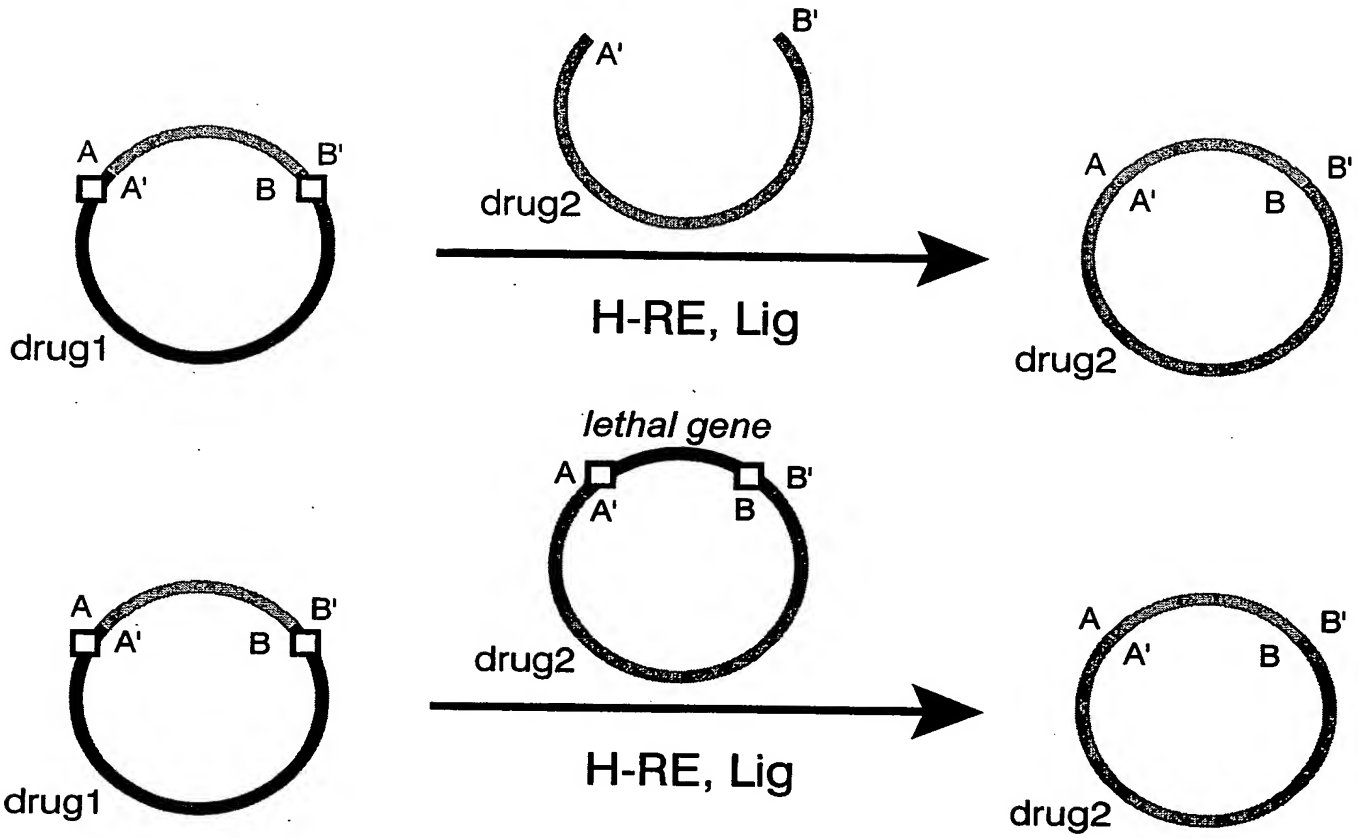


Fig 12

Sap I

How to make *Sap* I "one way"

- Methylases
- Orientation of sites in vector backbone in Donor Vector and in Acceptor Vector
- Lethal genes in stuffer fragments
- *Ear* I, not *Sap* I sites, in Acceptor Vectors

```
G C T C T T C N^N N N
C G A G A A G N N N N^

C T C T T C N^N N N
G A G A A G N N N N^
```

Key Advantage of *Sap* I

- Only three bases per exchange site left in Acceptor Vector

Fig. 13

Two Enzyme Approach

Sgf I – infrequent cutter of human cDNAs, two base 3' overhang

```
G C G A T^C G C
C G C^T A G C G
```

Pme I – infrequent cutter, blunt end cutter

```
G T T T^A A A C
C A A A^T T T G
```

FIG1A

Two Enzyme Approach

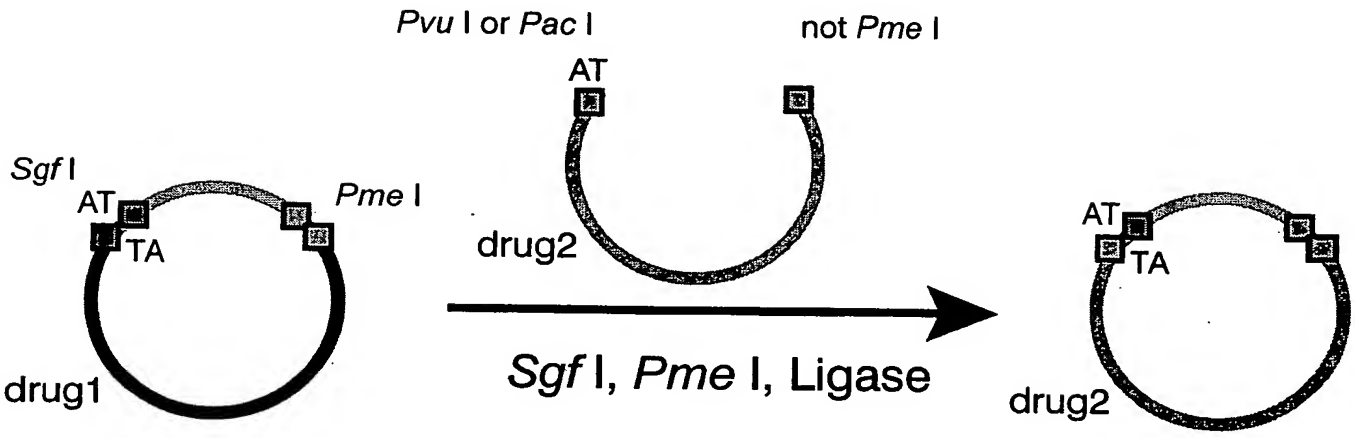
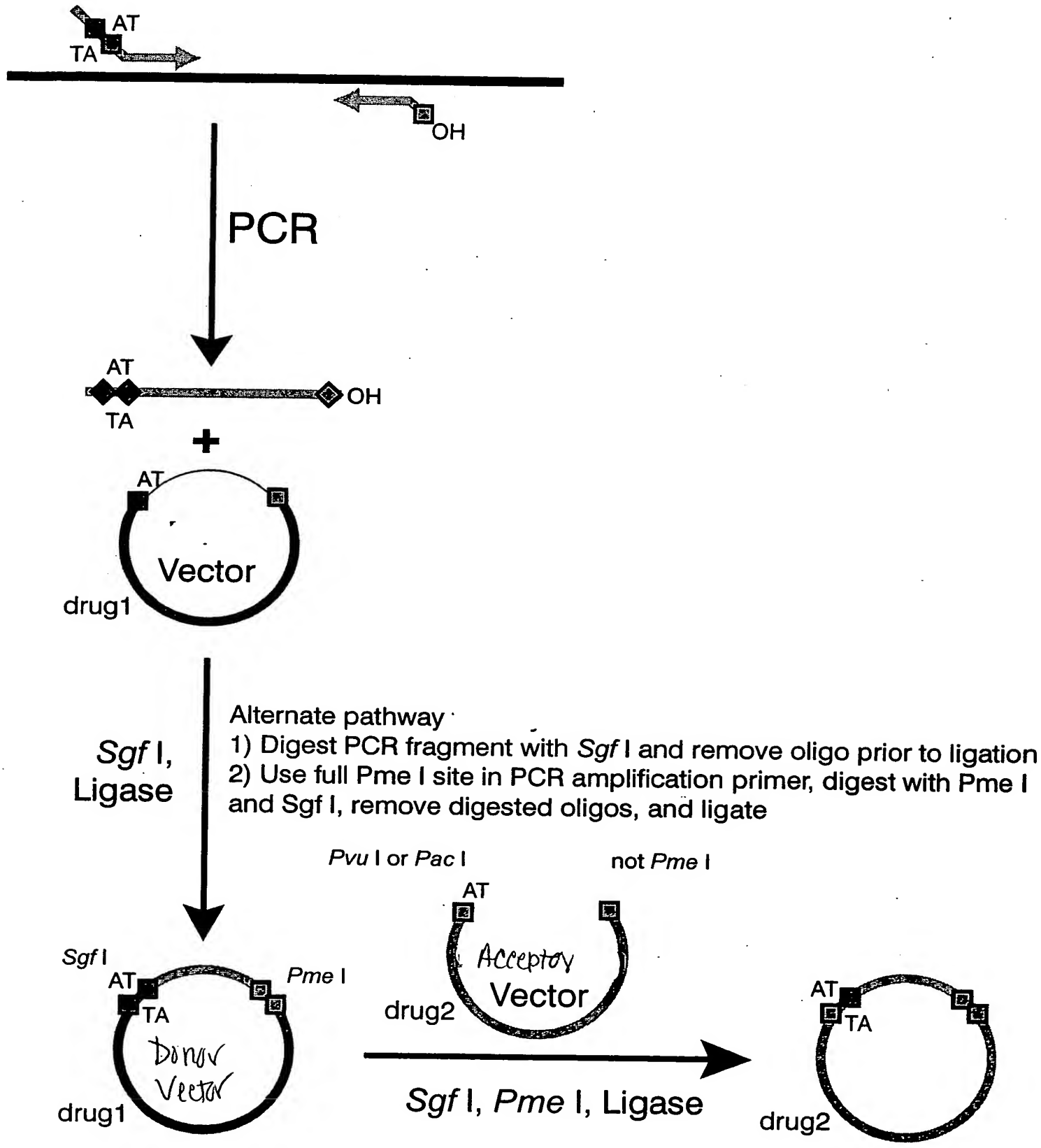


FIG 1AB

Two Enzyme Cloning Pathway with PCR Entry



N-terminal *Sgf* I site can allow N-terminal fusions OR *NO fusion*

NAAGGAGCGATCGCCATGg

--RBS- Kozak--

VAAGGAGCGATCGCCATG

KEQGlyAlaIleAlaMet

Fig. 1b

C-terminal *Pme* I site allows termination (+1AA) or C-terminal fusions

NNNGTTTAAACN

XaaValTer

NNNGTTTATCN with *Eco*RV

XaaValTyr

NNNGTTTCCAN with *Bal*I, etc.

XaaValSer

Fig 17

Coexpression Variation

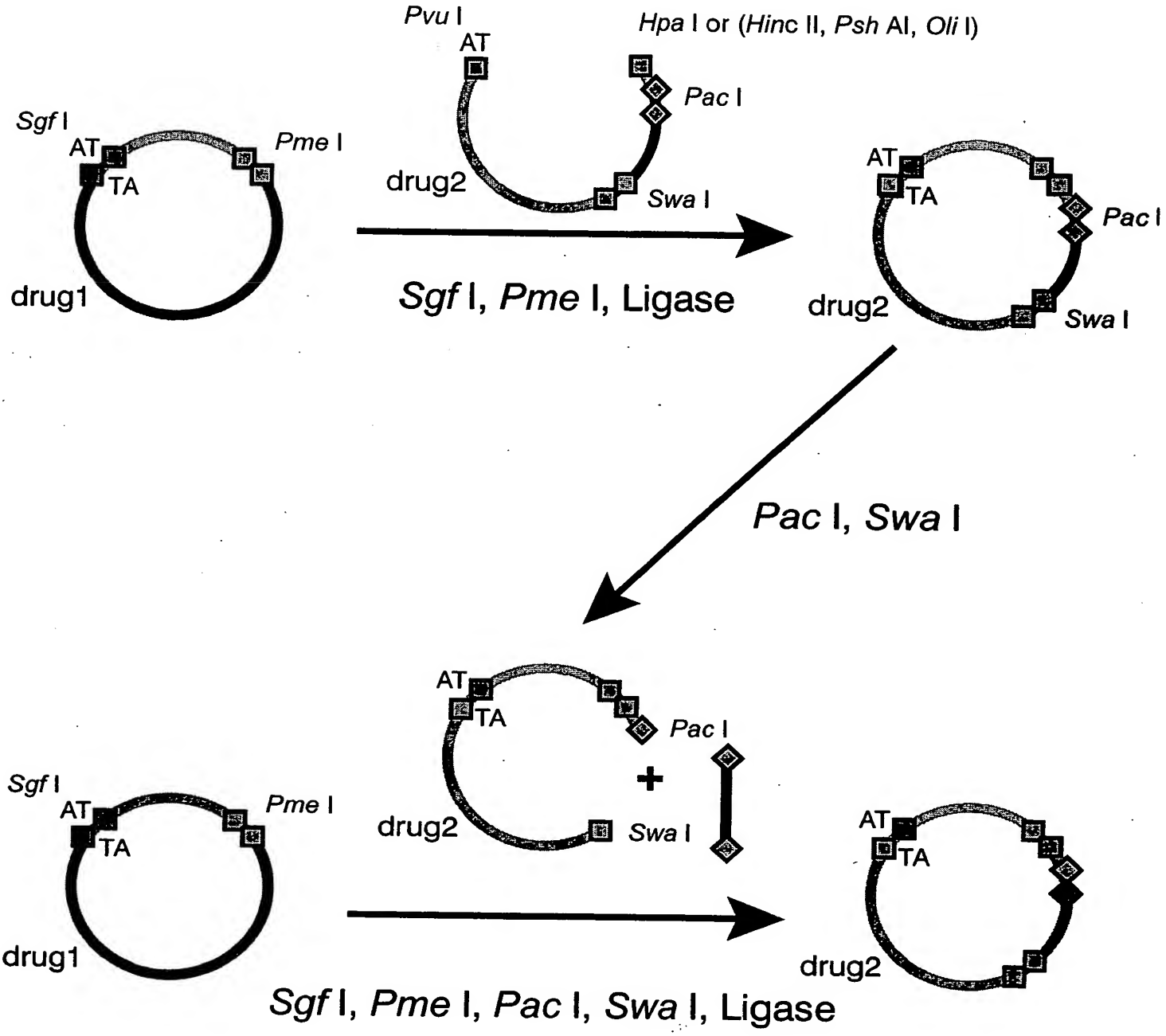


Fig 18

N-terminal *Pac* I--*Sgf* I fusion site

NAAGGATTAATCGCCATGg

KEQGlyLeuIleAlaMet

C-terminal *Pme* I--*Swa* I fusion site

NNNGTTTAAATN

XaaValTer

Fig 19A

N-terminal *Pac* I--*Sgf* I fusion site

NAAGGATTAATCGCCATGg

--RBS- Kozak--

C-terminal *Pme* I--*Swa* I fusion site

NNNGTTTAAATN

XaaValTer

Fig 19B

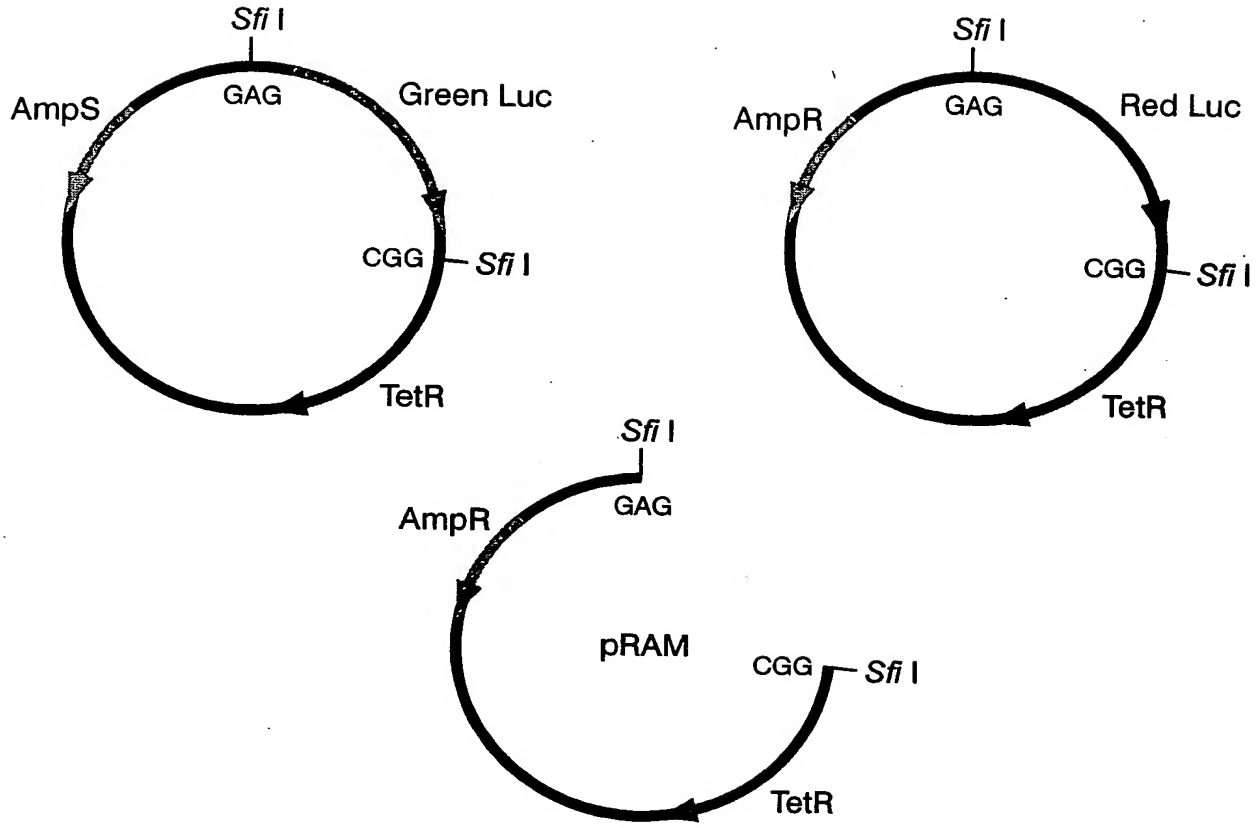
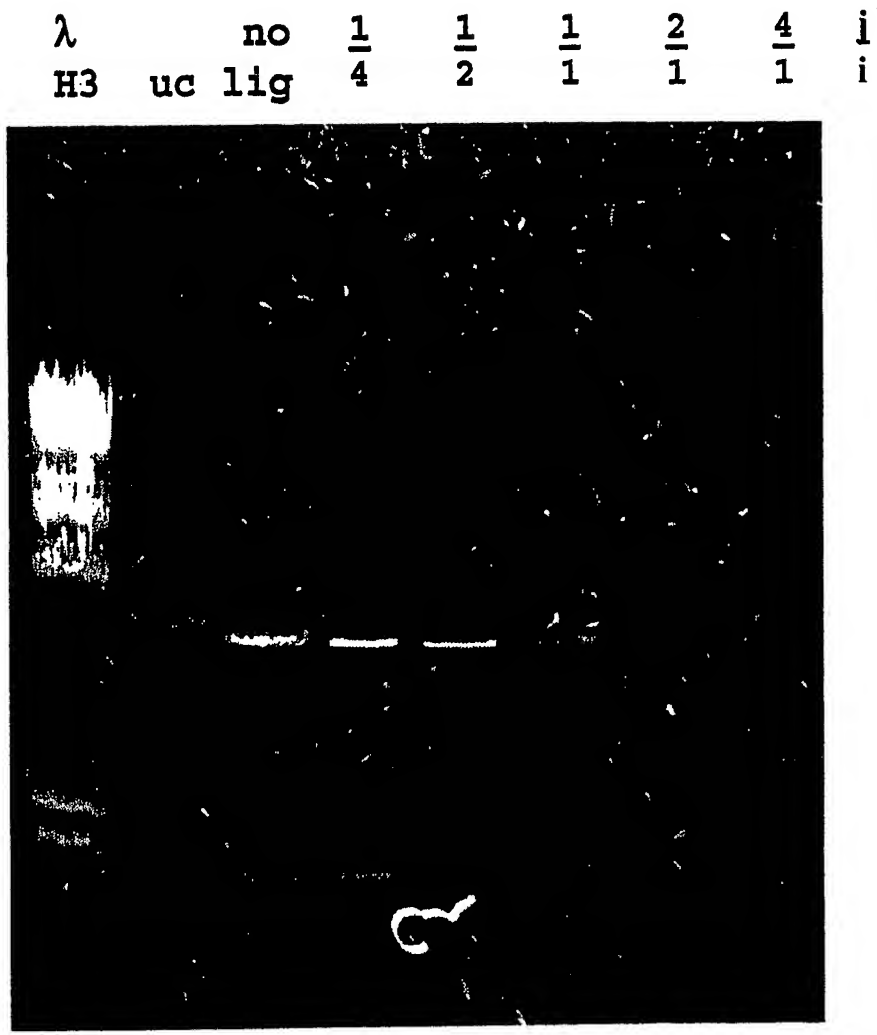

Sfi I
AmpS
GAG
Green Luc
CGG
Sfi I
TetR
Sfi I
AmpR
GAG
Red Luc
CGG
Sfi I
TetR
Sfi I
AmpR
GAG
pRAM
CGG
Sfi I
TetR

Fig20A

Sfi I cuts in ligase buffer, and cut ends religate with T4 DNA ligase



$i=N_0Mx10^3$ ends/ml for non-identical cohesive termini
$j=j\lambda(MW\lambda/MW)^{3/2}$ ends/ml

i=total concentration of DNA termini
j=effective concentration of one end of a DNA molecule in the immediate neighborhood of the other end of the same molecule

Note: *Sfi* I cut at 50°C; ligase at 22°C

Fig 20B

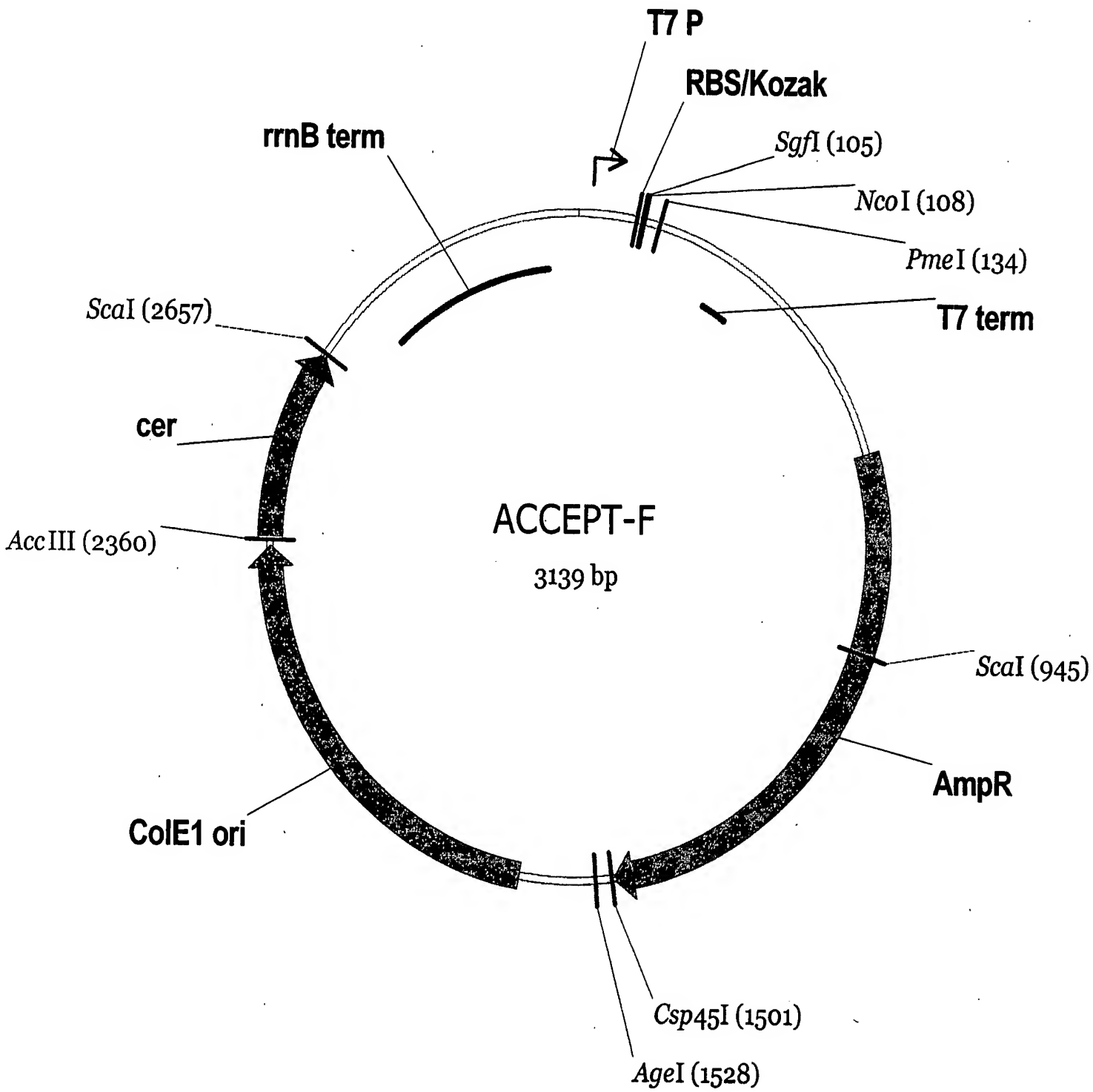
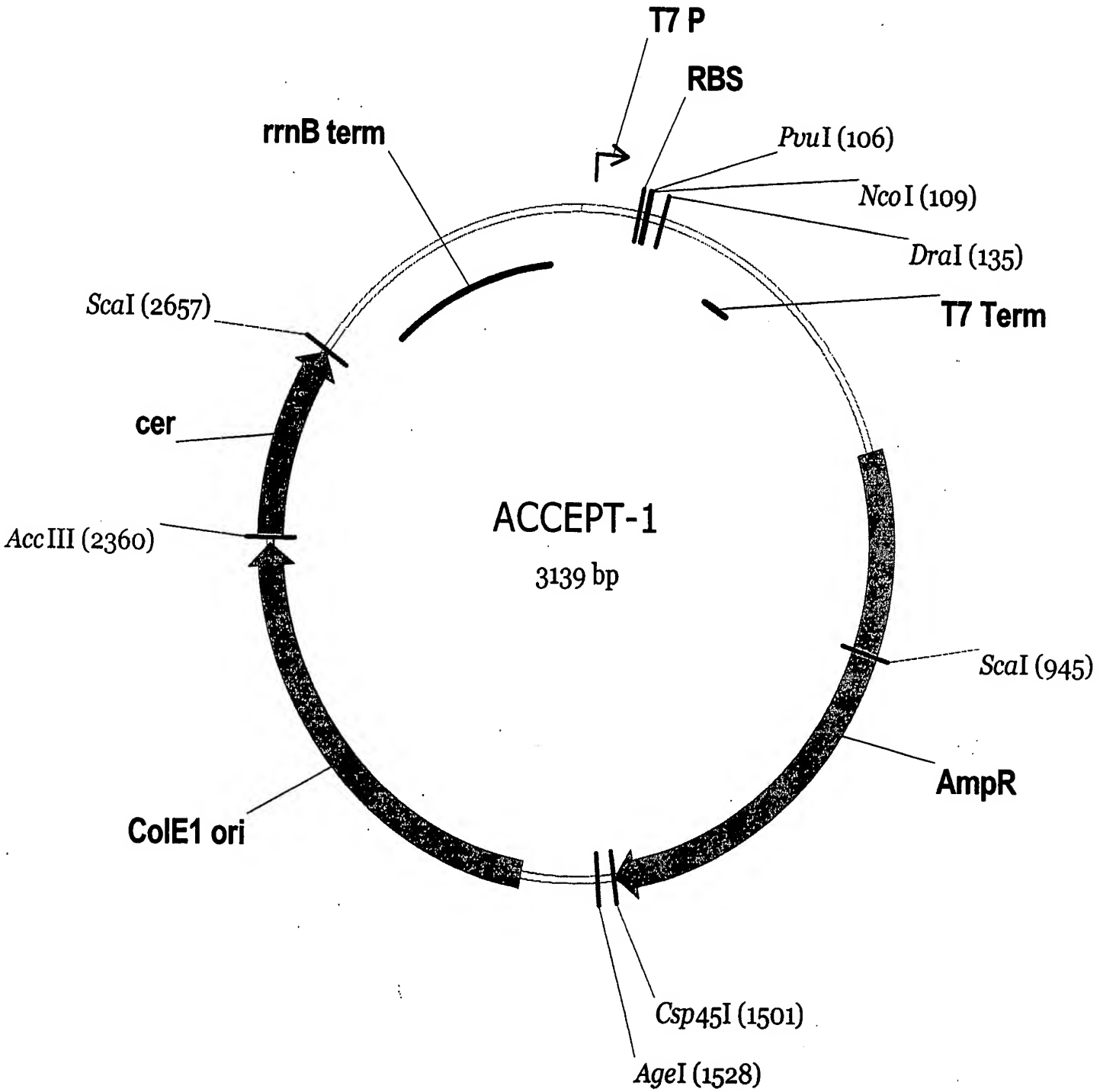
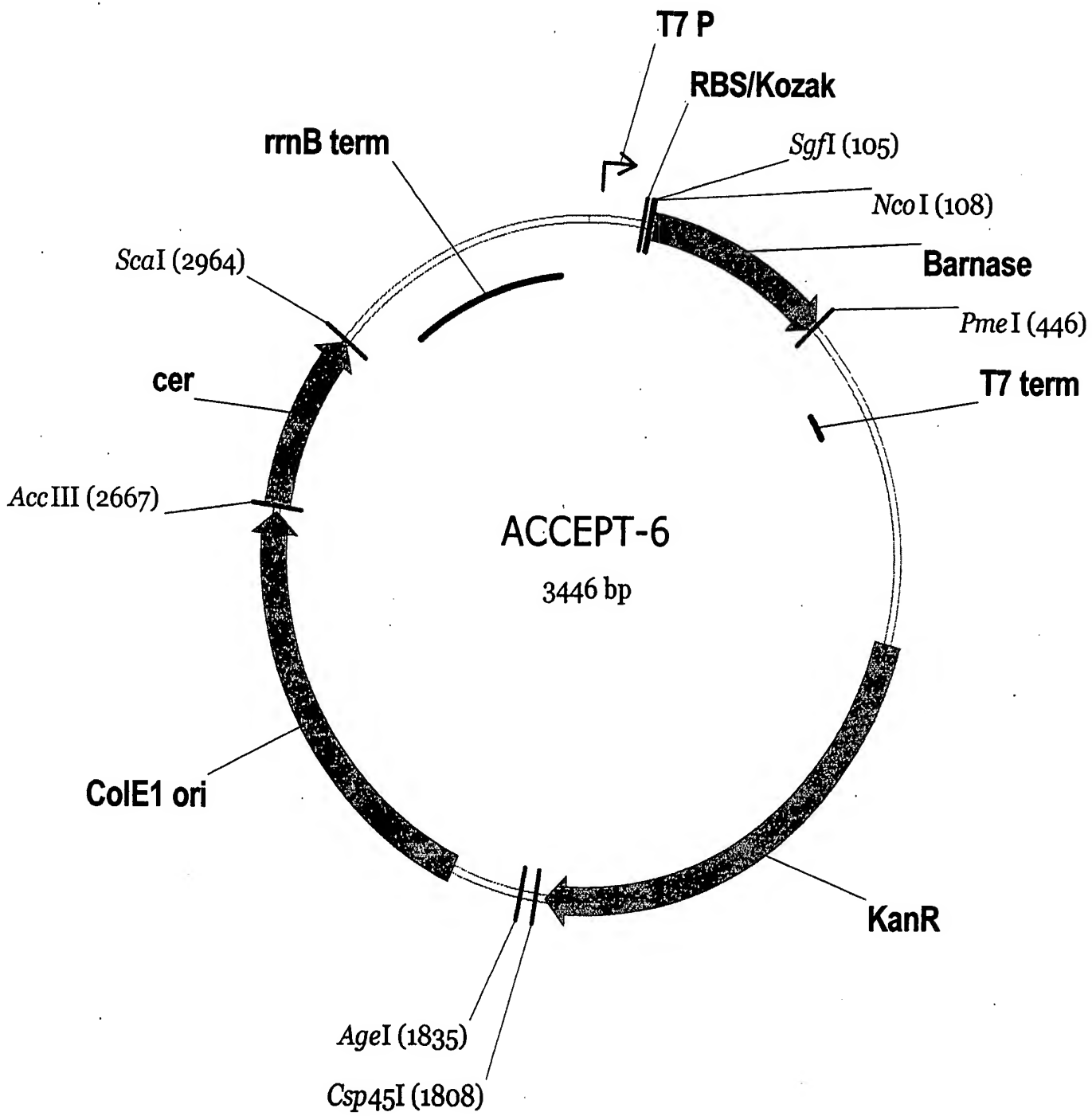
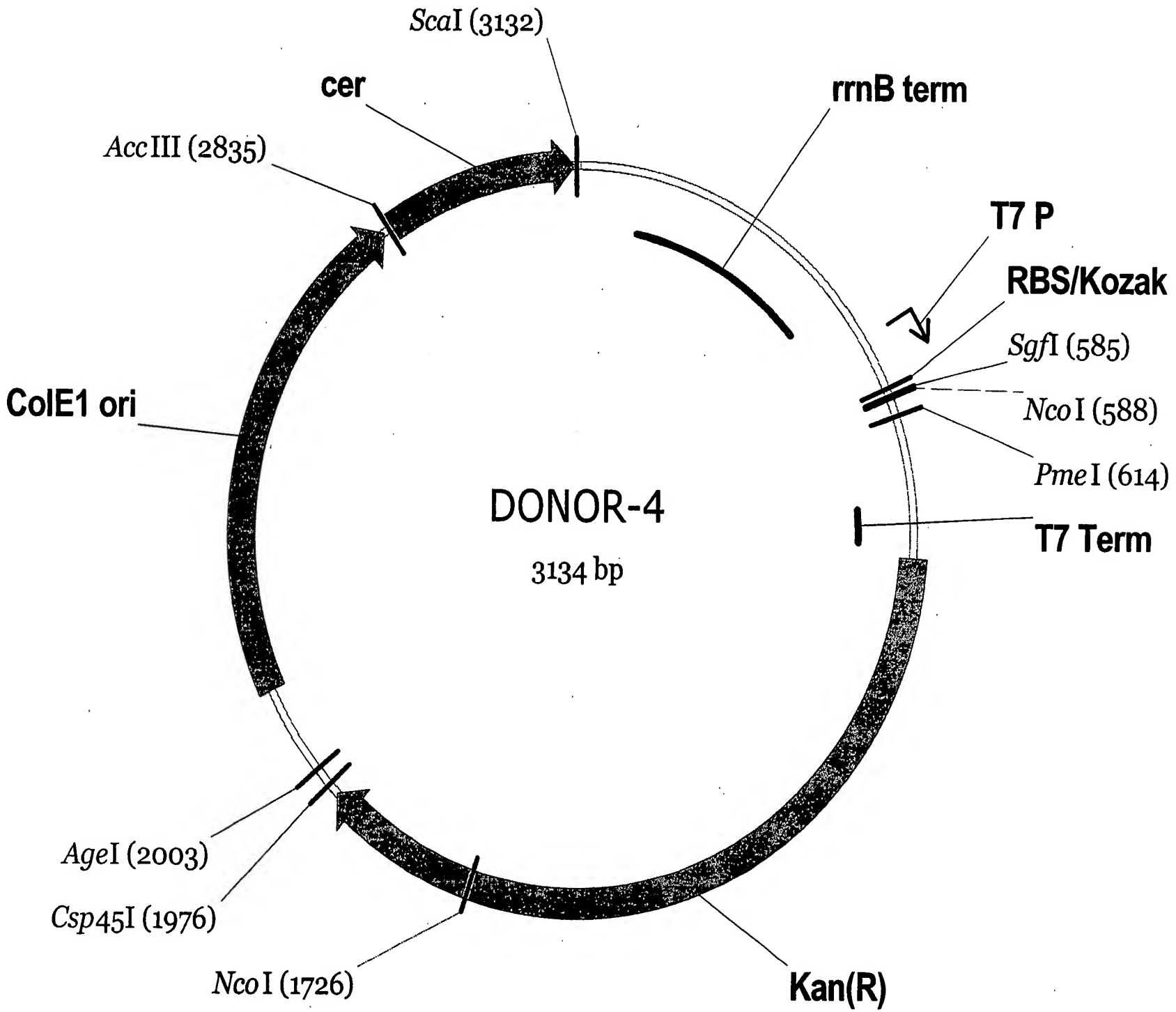
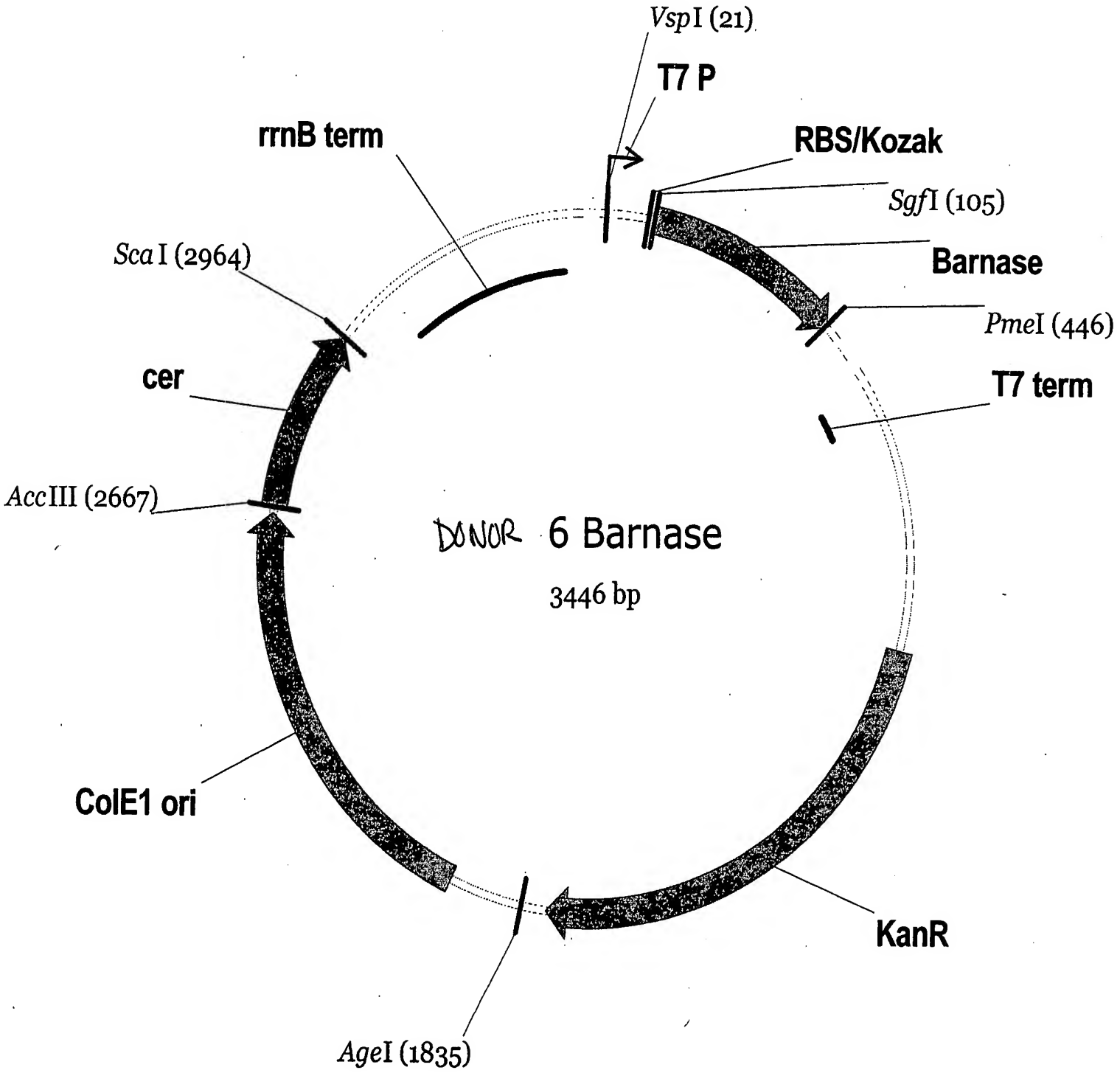

T7 P
RBS/Kozak
rrnB term
SgfI (105)
NcoI (108)
PmeI (134)
ScaI (2657)
T7 term
cer
ACCEPT-F
3139 bp
AccIII (2360)
ScaI (945)
AmpR
ColE1 ori
Csp45I (1501)
AgeI (1528)

Fig 21A-E


T7 P
RBS
rrnB term
PvuI (106)
NcoI (109)
DraI (135)
T7 Term
ScaI (2657)
cer
ACCEPT-1
3139 bp
AccIII (2360)
ScaI (945)
AmpR
ColE1 ori
Csp45I (1501)
AgeI (1528)


T7 P
RBS/Kozak
rrnB term
SgfI (105)
NcoI (108)
ScaI (2964)
Barnase
PmeI (446)
cer
T7 term
AccIII (2667)
ACCEPT-6
3446 bp
ColE1 ori
KanR
AgeI (1835)
Csp45I (1808)


ScaI (3132)
cer
rrnB term
AccIII (2835)
T7 P
RBS/Kozak
SgfI (585)
NcoI (588)
ColE1 ori
PmeI (614)
DONOR-4
3134 bp
T7 Term
AgeI (2003)
Csp45I (1976)
NcoI (1726)
Kan(R)


VspI (21)
T7 P
RBS/Kozak
SgfI (105)
Barnase
PmeI (446)
T7 term
rrnB term
ScaI (2964)
cer
AccIII (2667)
DONOR 6 Barnase
3446 bp
ColE1 ori
KanR
AgeI (1835)

Luciferase Expression in 3 Hosts

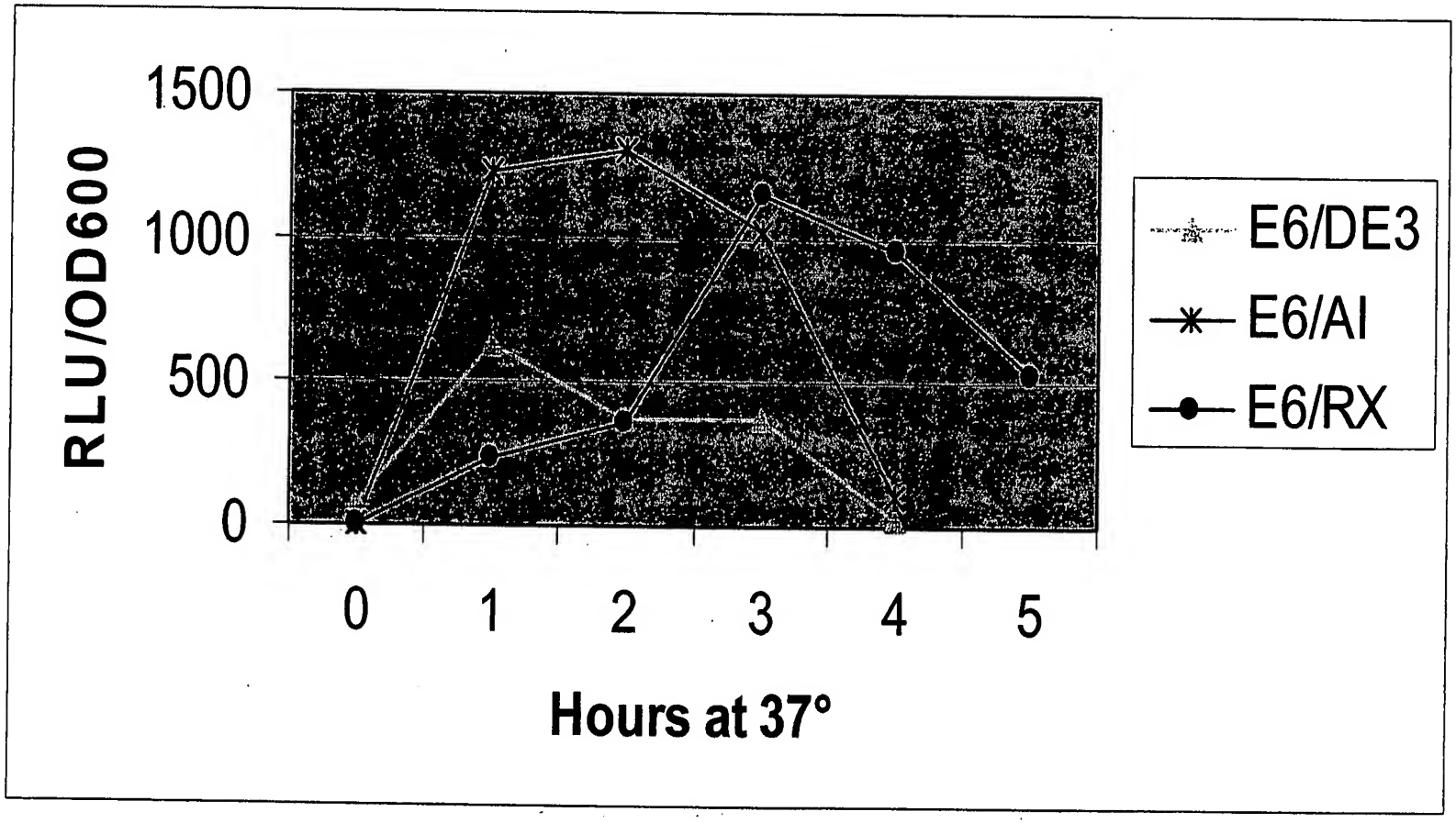
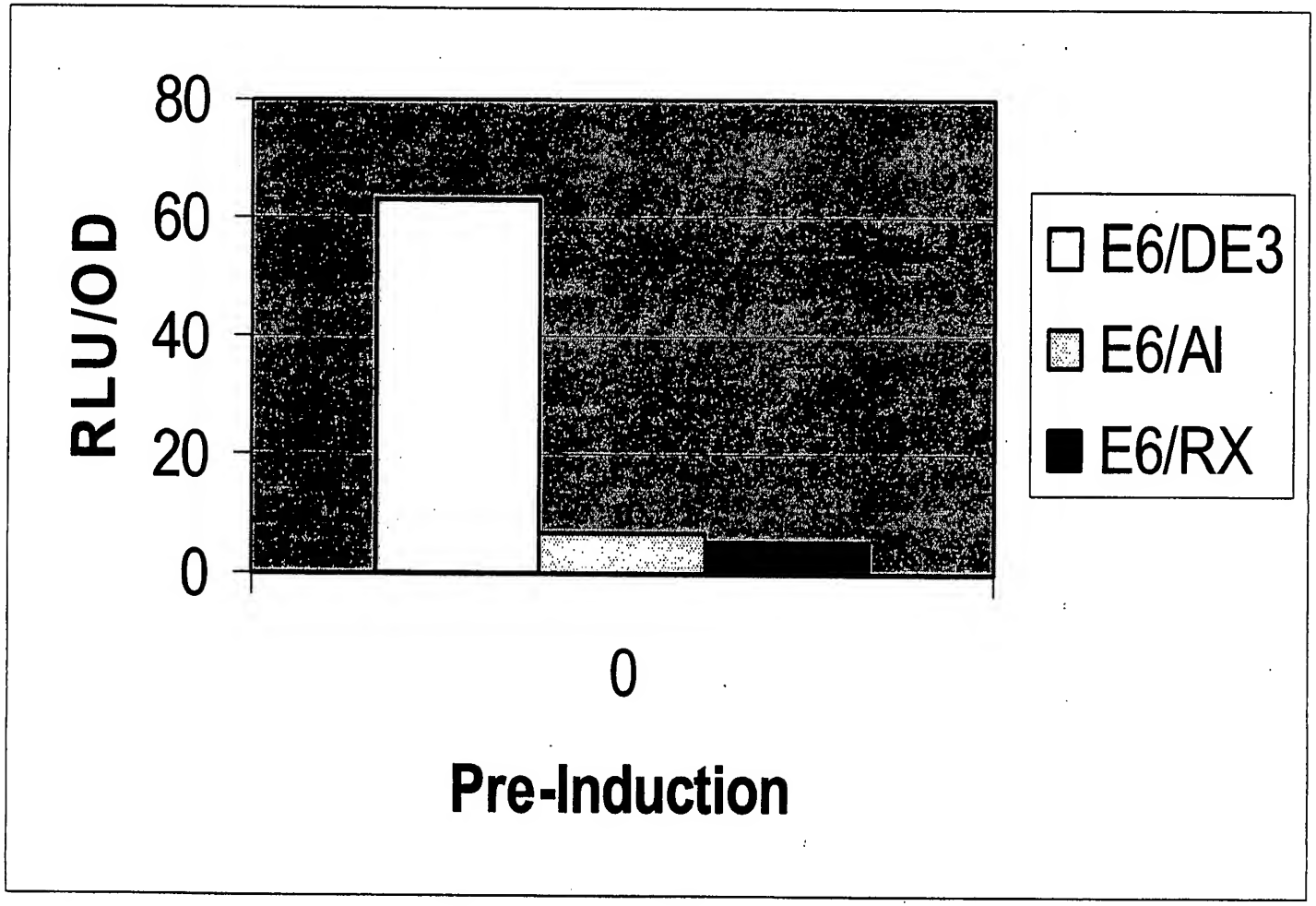


Fig 22A-C

Luciferase Expression in 3 Hosts at 25°C



Luciferase Expression in 3 Hosts at 25°C